

## Technical note

## Implications of the pH and temperature of diluted, cooled boar semen on fresh and frozen-thawed sperm motility characteristics

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Received 1 October 2009; received in revised form 15 April 2010; accepted 24 April 2010

## Abstract

Boar semen is typically collected, diluted and cooled for AI use over numerous days, or frozen immediately after shipping to capable laboratories. The storage temperature and pH of the diluted, cooled boar semen could influence the fertility of boar sperm. Therefore, the purpose of this study was to determine the effects of pH and storage temperature on fresh and frozen-thawed boar sperm motility end points. Semen samples ( $n = 199$ ) were collected, diluted, cooled and shipped overnight to the National Animal Germplasm Program laboratory for freezing and analysis from four boar stud facilities. The temperature, pH and motility characteristics, determined using computer automated semen analysis, were measured at arrival. Samples were then cryopreserved and post-thaw motility determined. The commercial stud was a significant source of variation for mean semen temperature and pH, as well as total and progressive motility, and numerous other sperm motility characteristics. Based on multiple regression analysis, pH was not a significant source of variation for fresh or frozen-thawed boar sperm motility end points. However, significant models were derived which demonstrated that storage temperature, boar, and the commercial stud influenced sperm motility end points and the potential success for surviving cryopreservation. We inferred that maintaining cooled boar semen at approximately 16 °C during storage will result in higher fresh and frozen-thawed boar sperm quality, which should result in greater fertility.

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**Keywords:** Spermatozoa; pH; CASA; Temperature; CASA; Sperm motility; Swine

## 1. Introduction

Boar semen can typically be cooled to 15–18 °C and held for numerous days in liquid form for use in arti-

ficial insemination [1–3], or transported to laboratories for cryopreservation [4]. Our initial results (unpublished) indicated that even though samples were diluted with shipping extender according to the manufacturer's instructions and our shipping protocol, the samples still arrived at our laboratory at varying temperatures and pHs.

It is well documented that pH and temperature are capable of influencing boar sperm physiology [1–3]. Indeed, when sperm samples are maintained outside of an optimal storage temperature range, the quality decreases [2,5,6]. What is not known, however, is the ability of pre-freeze pH and temperature to alter post-thaw quality, particularly when the semen is held (~24

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Table 1

Differences among studs in characteristics of boar semen samples (least square means) after collection, dilution, cooling to 15 °C, and maintenance at this temperature for 24 h.

	Stud				SEM	P
	1	2	3	4		
Temperature (°C)	16.5 <sup>a</sup>	17.0 <sup>a</sup>	19.1 <sup>b</sup>	15.1	0.2	0.0001
pH	6.84 <sup>a</sup>	6.68 <sup>b</sup>	6.68 <sup>b</sup>	6.75	.01	.02
Motility (%)	75 <sup>a</sup>	80 <sup>b</sup>	57	78 <sup>ab</sup>	0.8	.0002
Progressive Motility (%)	36 <sup>a</sup>	43 <sup>b</sup>	26	37 <sup>a</sup>	0.8	.0001
VAP (um/s)	87.0 <sup>a</sup>	96.6 <sup>b</sup>	71.8	91.3 <sup>ab</sup>	1.1	.009
VSL (um/s)	48.5	55.6	43.7	48.1	0.6	.06
VCL (um/s)	182.9 <sup>a</sup>	198.0 <sup>b</sup>	155.8	193.1 <sup>ab</sup>	1.9	.01
Lateral head amplitude (um)	8.6 <sup>a</sup>	8.7 <sup>a</sup>	7.8 <sup>b</sup>	8.5 <sup>a</sup>	.04	.006
Area (μm <sup>2</sup> )	9.8	10.1	9.3	9.7	0.1	.95
Linearity (%)	28.1 <sup>a</sup>	29.7 <sup>a</sup>	19.1 <sup>b</sup>	24.6	0.2	.0001
BCF (Hz)	31.0 <sup>a</sup>	30.7 <sup>a</sup>	33.6 <sup>b</sup>	30.9 <sup>a</sup>	0.2	.006
Elongation (%)	52.9 <sup>ab</sup>	53.5 <sup>ab</sup>	50.7	52.2 <sup>a</sup>	0.2	.003

<sup>a</sup> Within a row, values without a common superscript differed significantly.

<sup>b</sup> VAP: Path velocity; VSL: Progressive velocity; VCL: Track speed; BCF: Beat cross frequency.

h) prior to cryopreservation; this could occur when semen is transported overnight to a laboratory to perform the freezing. This is particularly important for genbanking activities, where semen samples are routinely collected at one location and shipped to a central laboratory for processing and storage. Therefore, the objective of the present study was to determine whether pH and temperature affected post-thaw motility characteristics of boar sperm that had been held for 24 h at approximately 15 °C prior to freezing, using data obtained retrospectively from samples arriving for cryopreservation at the USDA-ARS-NCGRP National Animal Germplasm Program laboratory.

## 2. Materials and methods

### 2.1. Sample collection and analysis

Semen (sperm-rich fractions) was collected from boars (two samples from 99 commercial composite boars and a single sample from one boar; N = 199) from four boar studs (Studs 1 through 4). The boars were fed a complete diet to meet all of their nutritional needs and had access to water ad libitum. All of the boars were 3 to 4 y of age and semen was collected once every 7 d. The studs were provided with the NAGP protocol [4] for diluting and cooling boar semen and the samples were processed as follows. Each semen sample was diluted with 37 °C An-

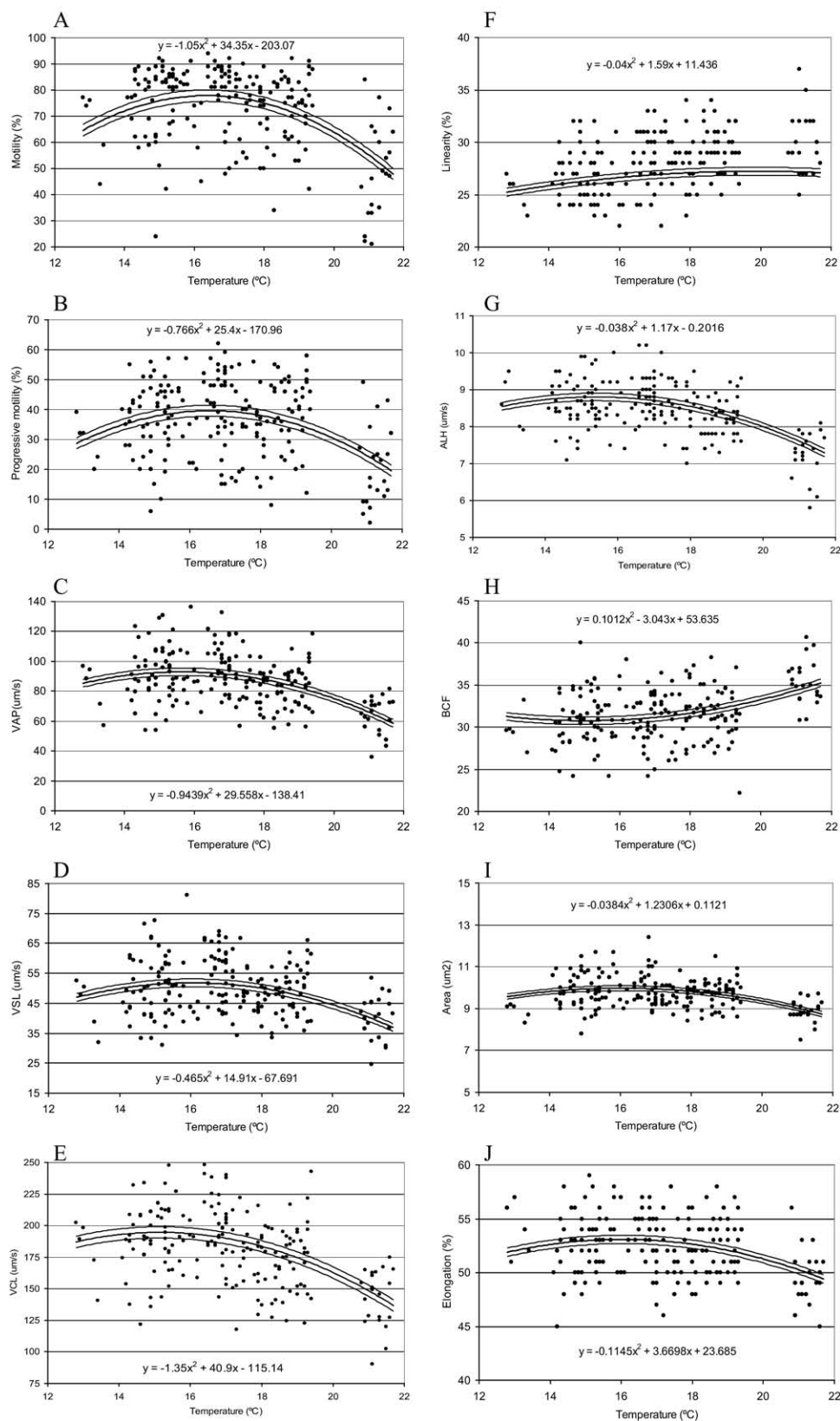
Table 2

Differences among studs in post-thaw characteristics of boar semen samples (least square means) after collection, dilution, cooling to 15 °C, and maintenance at this temperature for 24 h.

	Stud				SEM	P
	1	2	3	4		
Motility (%)	19 <sup>a</sup>	47 <sup>b</sup>	20 <sup>a</sup>	28	0.9	.0001
Progressive Motility (%)	7 <sup>a</sup>	24 <sup>b</sup>	7 <sup>a</sup>	10 <sup>a</sup>	0.6	.0001
VAP (um/s)	68.1 <sup>a</sup>	71.8 <sup>b</sup>	57.9	65.9 <sup>a</sup>	0.7	.0001
VSL (um/s)	50.7 <sup>a</sup>	56.2 <sup>b</sup>	46.2	49.6 <sup>a</sup>	0.6	.0001
VCL (um/s)	131.4 <sup>a</sup>	130.0 <sup>a</sup>	109.4 <sup>b</sup>	129.0 <sup>a</sup>	1.1	.0001
Lateral head amplitude (um)	6.8	6.2	5.8	6.3	0.1	0.2
Area (μm <sup>2</sup> )	8.6	9.6	8.8	9.1	.05	0.2
Linearity (%)	33.5 <sup>b</sup>	36.4 <sup>a</sup>	36.4 <sup>a</sup>	32.6 <sup>b</sup>	0.4	.003
BCF (Hz)	34.7 <sup>ab</sup>	34.0 <sup>a</sup>	35.6 <sup>ab</sup>	38.3	0.2	.03
Elongation (%)	48.6 <sup>b</sup>	51.5y <sup>a</sup>	50.5 <sup>b</sup>	48.6 <sup>b</sup>	0.3	.29

<sup>a</sup> Within a row, values without a common superscript differed significantly.

<sup>b</sup> VAP: Path velocity; VSL: Progressive velocity; VCL: Track speed; BCF: Beat cross frequency.



drohep Plus (295 mOsm; Minitube of America, Verona, WI, USA) 1:4 (v:v) in 50 mL centrifuge tubes, cooled to 23 °C over 1 h, and then cooled to 15 °C over an additional 1.5 h. The samples were placed in insulated boxes to maintain the temperature at 15 °C, and shipped to the laboratory overnight.

Upon arrival, pH and temperature of the samples was determined using a Corning 314 pH/Temperature Plus (Corning, Ithaca, NY, USA). The unit was calibrated according to the manufacturer's instructions before shipments were received. Three point calibration was performed using buffers of pH 4.0, 7.0, and 10.0 (Corning) which were cooled to 15 °C. The samples were then centrifuged (800 x g for 10 min at 15 °C), the supernatant was removed, and the sperm concentration from the resulting pellet determined using a spectrophotometer (Spermacue, Minitube, Minneapolis, MN, USA), specifically calibrated for boar sperm.

## 2.2. Boar semen cryopreservation

Boar sperm samples were diluted in 50 mL tubes using BF5 [7] cooling extender (CE; 15 °C; 52 mM TES, 16.5 mM Tris [hydroxymethyl] aminomethane, 178 mM glucose, 20% egg yolk; 325 mOsm) to 300 x 10<sup>9</sup> sperm/mL, and cooled to 5 °C over 2.5 h in a beaker containing water initially at 15 °C. The samples were then diluted drop-wise with BF5 freezing extender (91.5% CE, 6% glycerol, 2.5% Equex Paste, 1450 mOsm; Minitube of America) so that the final sperm concentration was 200 x 10<sup>9</sup> sperm/mL and loaded into 0.5 ml CBS straws (IMV Corporation, Minneapolis, MN, USA). The samples were frozen in liquid nitrogen vapor using a Minidigitcool UJ400 programmable freezer (IMV Corporation, Minneapolis, MN, USA) and a defined freeze curve (5 to -8 °C at 20 °C/min; -8 to -120 °C at 69 °C/min; and -120 to -140 °C at 20 °C/min). The straws were then plunged into liquid nitrogen for storage. Samples were thawed using a 50 °C water bath and agitating the straw for 20 s.

## 2.3. Motility analyses

The sperm motility was determined using computer automated semen analysis (CASA; Hamilton Thorne Motility Analyzer, Beverly, MA, USA). The CASA end points were as follows: 30 frames acquired, frame rate of 60Hz, minimum contrast of 55, minimum cell

size of five pixels, VAP cutoff of 20  $\mu$ m, progressive minimum VAP cutoff of 45  $\mu$ m/s, VSL cutoff of 5  $\mu$ m/s, static head size of 0.53–4.45, and magnification of 1.89. A minimum of seven fields and 1000 sperm were analyzed for each sample. All samples had total motility of  $\geq 70\%$ , determined immediately after collection using phase-contrast microscopy. Motility was analyzed for fresh samples immediately after centrifugation to remove the Androhep Plus diluent and for the frozen-thawed samples after thawing as described previously. For motility analysis, samples were brought to 37 °C and analyzed 5–7 min later. All boar sperm samples had an initial pre-freeze total motility of  $\geq 70\%$  determined using CASA.

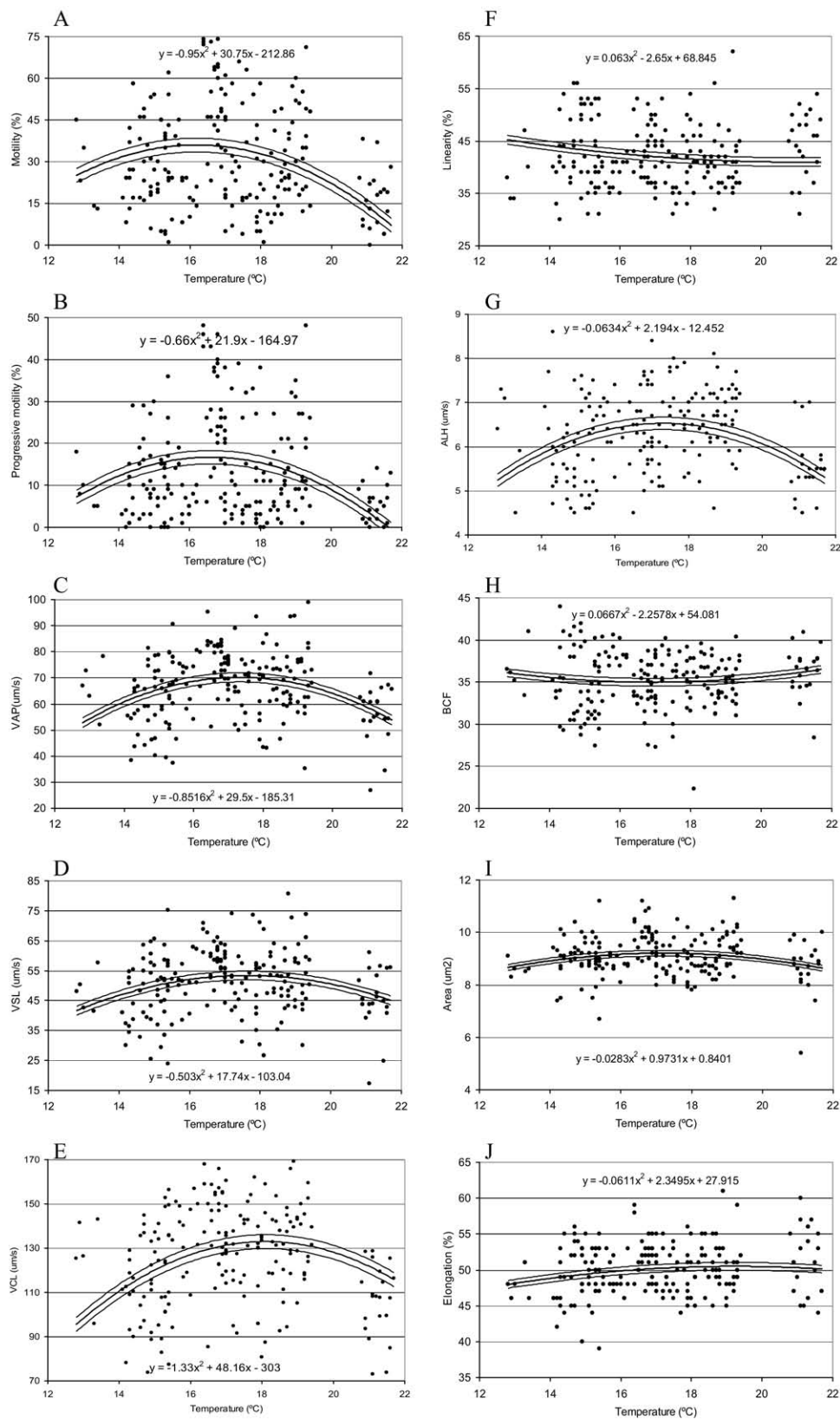
## 2.4. Statistics

The general linear model ANOVA was used to identify differences in boar sperm motility characteristics, sample temperature, and pH using commercial stud as the main effect [8]. The PDIF option of SAS [8] was used to identify the differences in the least-square means. Fresh and post-thaw motility measures were regressed upon a multiple regression model that included pH, temperature, commercial stud, boar within stud, and the appropriate second order polynomials (temperature\*temperature; pH\*pH) [8]. Sample pH was not a significant source of variation and was removed from the model.

## 3. Results

The mean temperature and pH of the samples were 17.2 °C (range 12.8–21.7) and 6.74 (range 5.91–7.14), respectively, however pH was not a significant source of variation it was not related to significant changes in diluted semen quality. Stud was a significant source of variation for temperature, pH, and all motility characteristics, except cell area for the fresh samples (Table 1). Stud was also a significant source of variation for many of the motility characteristics, when samples were analyzed following cryopreservation (Table 2). A multiple regression analysis that included temperature, stud, boar within stud, and the second order polynomial term for temperature, was predictive of the pre-freeze (Fig. 1;  $P < 0.0001$ ) and post-thaw (Fig. 2;  $P < 0.0002$ ) CASA motility characteristics. In both instances, fresh

Fig. 1. Results of multiple regression analyses of pre-freeze temperature and motility characteristics (CASA) of boar semen. For each graph, the actual data points, the regression line (solid), and CV for each regression line (dashed) are presented. Each model was significant ( $P < 0.0001$ ). VAP = Path velocity; VSL = Progressive velocity; VCL = Track speed; ALH = Lateral head amplitude; BCF = Beat cross frequency.



and post-thaw, a pre-freeze temperature of approximately 16–17 °C resulted in samples with the highest percentages of motile and progressively motile sperm (Fig. 1 and 2). Likewise, this temperature range resulted in samples with the highest velocities (VAP, VSL and VCL) as well (Fig. 1 and 2). Furthermore, significant models were also derived using the fresh and post-thaw data which described the type of motion (linearity, ALH and BCF), as well as the size and shape (area and elongation) of boar sperm (Fig. 1 and 2).

#### 4. Discussion

The means in which the pH and temperature influence the sperm are well documented. The intracellular pH of the samples may be driven by numerous extracellular stimuli; consequently, the storage medium is capable of exerting tremendous influence on diluted sperm. Cross [9] demonstrated that storing sperm in media with increasing pH resulted in an increase in intracellular pH and consequently an increasing incidence of acrosomal responsiveness. How the samples are stored is also important, as large volumes of air in the storage vessels may decrease the total number of motile sperm over time and increase the pH of the diluent [3]. In diluents which contain bicarbonate buffering systems, such as the Androhep Plus which was used in this study, CO<sub>2</sub> travels down a concentration gradient from the diluent into the ambient air in the tube, thus simultaneously removing protons [3]. This event increases the pH of the diluent, which increases the mitochondrial activity of the sperm, which in turn shortens their lifespan [3]. Under *in vivo* conditions, the increase in pH is a normal, required event where during capacitation intracellular pH increases followed by an additional increase during the acrosome reaction, which also is needed for inducing hyperactivation [10–12]. However, premature induction of these processes will decrease sample quality, and consequently, fertility. Although significant differences in stud pH were detected, it was not surprising that this did not influence the quality of the samples, considering that the semen was diluted in a defined medium and considering the range of mean pH (Table 1).

Similarly, temperature can also affect the quality of

a sample. Diluted samples can typically be held at low temperatures and used for multiple days while still producing acceptable fertility [1,5,6]. However, storage outside an acceptable temperature (~16 °C) decreased the percentage and quality of motile sperm (Fig. 1 and 2) plasma membrane integrity, the percentage of acrosome intact sperm [2], and increased lipid peroxidation [13]. Consequently, by controlling the temperature over the duration of storage, it should be possible to avoid these negative side effects.

Based on the present study, there was an optimal pre-freeze holding temperature (approximately 16 °C). In order to maximize motility during pre-freeze storage and following cryopreservation, the 16 °C temperature must be maintained (Fig. 1), or decreases in total motility and progressive motility as well as cell velocities (Fig. 1 and 2) occurred. Although samples analyzed in this study were not intentionally induced to capacitate, acrosome react and hyperactivate the motility and swimming patterns do suggest that the processes of diluting, holding and cryopreserving sperm may be responsible for inflicting damage severe enough to induce these processes. Consequently, each step of the collection, dilution, holding, and cryopreservation process must be monitored to optimize post-thaw success.

In conclusion, the present study demonstrated the importance of properly diluting and cooling boar semen, regardless of whether the samples will be used for cooled liquid insemination or following cryopreservation. Improperly treating samples may result in initiation of capacitation and acrosomal exocytosis. Perhaps this can be induced in our cooling and shipping system by improper preparation of the dilution medium, not properly preparing the shipping container to maintain the temperature, exposure of the shipping container to excessive (too hot or cold) external temperatures during shipping, or incorrect filling of the sample tubes [3]. Furthermore, based on the present study, there are two important issues for the boar stud industry. First, it is important to monitor the quality control of the samples from collection, through dilution and storage, cryopreservation, and insemination. Even slight shifts in temperature can dramatically influence sample quality, regardless

Fig. 2. Results of multiple regression analyses of pre-freeze temperature and post-thaw motility characteristics (CASA) of boar semen. For each graph, the actual data points, the regression line (solid), and CV for each regression line (dashed) are presented. Each model was significant ( $P \leq 0.0002$ ). VAP = Path velocity; VSL = Progressive velocity; VCL = Track speed; ALH = Lateral head amplitude; BCF = Beat cross frequency.

of whether the samples will be inseminated in cooled, liquid form or following cryopreservation. Second, although our dilution and shipping protocol was explained to each of the studs, it was apparent that other factors (external temperatures, inattention to detail, etc.) contributed to the quality of the diluted semen. We inferred that this accounted for differences among studs (Table 1); however, maintaining more stringent quality control measures should enhance post-thaw quality.

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